REMARKS

The specification has been amended to change the title to one indicative of the invention to which the claims are directed, the trademark symbol has been incorporated where applicable and an abstract has been added in compliance with 37 C.F.R. 1.72(b) and 37 C.F.R. 1.121 as required. Addionally, claims 24, 27 and 29 have been amended and claims 25, 28 and 31 – 33 have been cancelled. No new matter is added by virtue of the amendments to the specification and claims and entry is respectfully requested.

The Examiner has objected to claims 24 to 30 under 35 U.S.C. 102(e) as being anticipated by Armitage et al. This objection is respectfully traversed in view of newly amended claims taken together with the comments provided below.

Applicant has amended the claims to indicate that the soluble recombinant human CD40L is an 18 KDa fragment, consisting of amino acid 108 to 261 of SEQ ID NO:1. Support for the amendment can be found throughout the disclosure as, for example, on page 8, lines 27, 32 and 33, where it is clearly indicated that the 18 KDa CD40L fragment is used.

It is first submitted that Armitage does not teach specifically the use of the 18 KDa fragment. Secondly, Armitage teaches that monomeric soluble CD40L are antagonists of CD40 (see column 10, lines 62 to 65). Armitage also teaches that oligomeric CD40L acts as a CD40 agonist (see column 10, line 61). Applicant respectfully submits that 18 KDa CD40L was known in the art, prior to the filing of this application, to be a homotrimer (oligomer) in solution. This is clearly shown and supported by a paper by Mazzei *et al.*, published in the Journal of Biological Chemistry on March 31, 1995 (copy provided herewith). Thus, it would have been obvious to one skilled in the art that the 18 KDa CD40L used in the present invention is an oligomer in solution. However, the present invention shows that 18 KDa CD40L inhibits alloimmune response and that therefore *oligomeric* 18 KDa CD40L is a CD40 *antagonist* which is contrary to the teaching of Armitage. Accordingly, the claims, as amended, are clearly not anticipated by Armitage *et al.*

The claims are further rejected under 35 U.S.C. 102 as being anticipated by Aruffo *et al.* Again, this objection is respectfully traversed. It is respectfully submitted that Aruffo et al. do not teach the inhibition of CD40L binding to CD40 with soluble CD40L, but rather uses soluble ligands of CD40L to prevent the interaction between T cells and B cells. Therefore, Aruffo does not anticipate the instant invention wherein 18 KDa CD40L is used directly. Furthermore, Aruffo does not teach the use of an 18KDa CD40L. Therefore, the amended claims are novel in view of Aruffo.

The Examiner has further objected to claims 24-30 under 35 U.S.C. 103(a) as being unpatentable over Armitage et al. and/or Aruffo et al. in view of Black et al. This objection is respectfully traversed.

As mentioned above, none of the cited references teach or suggest the use of 18KDa CD40L to inhibit an alloimmune response. Furthermore, the fact that the 18KDa CD40L exists as an oligomer and is a CD40 antagonist clearly distinguishes the invention from Armitage et al. since Armitage teaches that oligomers CD40L are agonists (i.e. activate B cells). As indicated above, Aruffo does not teach the use of a CD40L ligand, but rather a ligand to a CD40L to inhibit B cell activation which is very different from the instant invention. Therefore, Armitage et al. nor Aruffo et al. in combination with Black et al. suggest or make obvious the use of 18KDa CD40L to inhibit an alloimmune response.

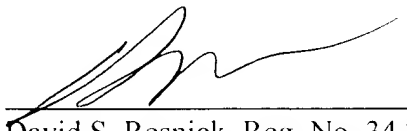
In the event that there are any questions relating to this Amendment or to the application in general, it is kindly requested that the Examiner contact the undersigned attorney concerning the same to expedite prosecution of this application.

FEE AUTHORIZATION

If any additional fees are associated with this submission, the Commissioner is authorized to charge fee deficiencies to the NIXON PEABODY LLP Deposit Account No. 50-0850. Any overpayments should be credited to said Deposit Account.

Date: May 19, 2003

Respectfully submitted,



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Recombinant Soluble Trimeric CD40 Ligand Is Biologically Active*

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From the Glaxo Institute for Molecular Biology, 14 Chemin des Aulx, 1228 Plan les Ouates, Geneva, Switzerland and §INSERM Unité 132, Hopital Necker-Enfants Malades, 149 Chemin de Sevres, 75743 Paris Cedex 15, France

CD40 ligand (CD40L) is expressed on the surface of activated CD4$^+$ T cells, basophils, and mast cells. Binding of C40L to its receptor, CD40, on the surface of B cells stimulates B cell proliferation, adhesion and differentiation. A preparation of soluble, recombinant CD40L (Tyr-45 to Leu-261), containing the full-length 29-kDa protein and two smaller fragments of 18 and 14 kDa, has been shown to induce differentiation of B cells derived either from normal donors or from patients with X-linked hyper-IgM syndrome (Durandy, A., Schiff, C., Bonnefoy, J.-Y., Forveille, M., Rousset, F., Mazzei, G., Milili, M., and Fischer, A. (1993) *Eur. J. Immunol.* 23, 2294–2299). We have now purified each of these fragments to homogeneity and show that only the 18-kDa fragment (identified as Glu-108 to Leu-261) is biologically active. When expressed in recombinant form, the 18-kDa protein exhibited full activity in B cell proliferation and differentiation assays, was able to rescue of B cells from apoptosis, and bound soluble CD40. Sucrose gradient sedimentation shows that the 18-kDa protein sediments as an apparent homotrimer, a result consistent with the proposed trimeric structure of CD40L. This demonstrates that a soluble CD40L can stimulate CD40 in a manner indistinguishable from the membrane-bound form of the protein.

CD40 ligand (CD40L, TRAP, or gp39) is a 39-kDa glycoprotein expressed as a type II integral membrane protein on the surface of T cells, basophils, and mast cells (1–5). The interaction of CD40L with CD40 in association with different cytokines (for reviews see Refs. 6 and 7) is required for B cell proliferation and for production of immunoglobulins. Mutations or deletions in the CD40L gene cause X-linked hyper-IgM syndrome, HIGM1[1] (for review, see Ref. 8). The majority of the CD40L mutations in HIGM1 patients are located in the extracellular domain of the CD40L, resulting in a failure of the ligand to bind CD40. Purified B cells from patients with X-linked hyper-IgM syndrome respond normally to agonistic anti-CD40 antibodies and recombinant CD40 ligand, indicating the lack of an active CD40L *in vivo* (9–11). The role of CD40L *in vivo* is supported as well by animal model studies using neutralizing antibodies specific for murine CD40L (muCD40L) that block both primary and secondary humoral response to T cell-dependent antigen (12–13). Together, these experimental studies and clinical results demonstrate the pivotal role of the CD40L-CD40 interaction in the regulation of humoral immunity (8, 12, 13).

Based on its structural homology with TNF (14), CD40L has been predicted to exist as a homotrimer in the cell membrane (15). The interaction of CD40L with B cells can be more readily investigated if the active site of CD40L can be produced in a soluble form. To obtain such molecule, we expressed in *Escherichia coli* the extracellular domain of CD40L (shuCD40L-EC: Tyr-45 to Leu-261). In addition to the expected full-length extracellular domain (29 kDa), two other CD40L fragments of 18 and 14 kDa were observed in *E. coli* extracts. This mixture of the CD40L fragments was shown to be active in a B cell differentiation assay. Following purification of the 29-, 18-, and 14-kDa fragments, only the 18-kDa fragment was able to recognize CD40, both in solution and on the surface of human B cells. The 18-kDa fragment showed biological activities similar to those described for the membrane-bound form of CD40L, including induction of B cell proliferation and differentiation, rescue of B cells from apoptosis, and binding to soluble CD40. The amino acid sequence of the 18-kDa fragment coincides in size and homology with the mature TNF molecule and behaves as a homotrimeric molecule.

MATERIALS AND METHODS

Construction of the Extracellular Domain of CD40L—The extracellular domains of human and murine CD40L were amplified by polymerase chain reaction from the full-length genes (1, 16), digested with *NdeI* and *XbaI* and cloned into p'286elta vector containing p_L (17) for expression in *E. coli*. All constructs were verified by DNA sequencing.

Purification of the Recombinant 29-kDa shuCD40L-EC and Fragments—The purification was performed as follows. Fifteen g of cell paste was resuspended in 50 ml of lysis buffer (50 mM Tris-HCl, pH 7.5, 25% (w/v) sucrose, 1 mM EDTA, 1 mM EGTA, 5 mM benzamidine-HCl, 1.5 mM phenylmethylsulfonyl fluoride, 0.5 mg/ml lysozyme, and 0.1% sodium azide) and incubated at room temperature for 60 min under mixing. The lysate was centrifuged at 27,000 × *g* for 60 min. CD40L-EC was recovered as an insoluble pellet. It was washed seven times with 200 ml of 20 mM Tris-HCl, pH 7.5, 2 mM EDTA, 2 mM EGTA, 1% (v/v) Triton X-100, 2 mM benzamidine-HCl, 1 μg/ml leupeptin, 1 μg/ml pepstatin, 1 mM phenylmethylsulfonyl fluoride, and 0.2 M NaCl. The washed pellet was solubilized in 20 ml of 25 mM triethylamine (pH 11.5) containing 7.5 M urea and 10 mM CHAPS, spun, and supernatant was loaded to a Q-Hyper D Column (2.6 × 5 cm, Sepracor S.A., France) precquilibrated with solubilization buffer. Bound proteins were eluted stepwise with a pH gradient of 25 mM triethylamine at pH 11, 25 mM triethylamine at pH 10, and 25 mM Tris-HCl, pH 8.5, containing 7.5 M urea (deionized with Amberlite AG501-X8) and 10 mM CHAPS. Protein peaks were analyzed by SDS-PAGE, pooled, and dialyzed against 5 liters of 0.5% (v/v) acetic acid.

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[1] The abbreviations used are: HIGM1, hyper-IgM syndrome; shuCD40L-EC, soluble human CD40 ligand extracellular domain; 18-kDa shuCD40L, 18-kDa soluble human CD40 ligand; smuCD40L, soluble murine CD40 ligand; TNF, tumor necrosis factor; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid; PBS, phosphate-buffered saline; IL, interleukin; PAGE, polyacrylamide gel electrophoresis.

Purification of the 18-kDa shuCD40L—Recombinant 18-kDa shuCD40L was purified as described above from 78 g of cell paste with minor modifications to ensure proper renaturation; washed pellet was extracted with 100 mM Tris-HCl, pH 7.5, 5 mM EDTA, 10 mM dithiothreitol, and 6 M guanidinium HCl, the 18-kDa monomers were separated on a Sephacryl S-200 (5 × 90 cm) equilibrated with 100 mM Tris-HCl, pH 7.5, 5 mM EDTA, 1 mM dithiothreitol, and 6 M guanidinium HCl. Monomers were pooled and desalted on a Sephadex G-25 (5 × 50 cm) equilibrated with 50 mM Tris-HCl, pH 9.8, and 7 M urea. The protein pool was loaded onto a Q-Hyper D column (5 × 4 cm) equilibrated with same buffer. The 18-kDa protein was eluted with the equilibration buffer containing 100 mM NaCl, and the buffer was exchanged on a Sephadex G-25 (5 × 50 cm) to 50 mM sodium phosphate, pH 6.5, containing 6 M urea. Renaturation was done by diluting the protein pool to 20 μg/ml and to 4 M urea with 50 mM phosphate buffer, pH 6.5, and then added dropwise to an equal volume of 50 mM sodium phosphate, pH 6.5, containing 0.1 mM reduced glutathione and 0.01 mM glutathione disulfite (redox buffer) plus 4 M urea and 2.5% (w/v) ammonium sulfate. Urea was dialyzed out stepwise by two subsequent changes of 60 liters of redox buffer. Glutathione was then removed by dialysis against several changes of 25 mM sodium phosphate, pH 6.5. Renaturated shuCD40L was concentrated and clarified by centrifugation, filtered through Millex 0.22-μm sterile filters (Millipore), and stored at −80 °C. All manipulations were done at 4 °C or as otherwise indicated.

sCD40/sCD40L Binding Assay—Purified 18-kDa shuCD40L was coated on a 96-well plate (Maxisorb, Nunc) and detected by incubating with sCD40-Fc (1) and then developed with goat anti-mouse Fc peroxidase-conjugated antibody. Specificity of binding was determined by competition with an excess of 18-kDa shuCD40L added with the soluble CD40-Fc. The binding assay is briefly as follows. The 18-kDa shuCD40L was diluted to a concentration of 60 μg/ml in 0.2 M sodium phosphate buffer, pH 7. Serial dilutions containing 100 μl/well were made and incubated overnight at 4 °C. The wells were then washed with phosphate-buffered saline, 0.05% (v/v) Tween 20 (TPBS) and blocked with 150 μl of PBS, 1% (v/v) normal goat serum and 2% (v/v) bovine serum albumin (NBPBS, for 1 h at 37 °C). After washing with TPBS, 100 μl of sCD40-Fc at (2 μg/ml diluted in NBPBS) was added. Following a 1-h incubation at 4 °C, bound CD40-Fc was detected by peroxidase-conjugated goat anti-mouse antibody.

Transfection of the Human CD40L in COS-7 Cells—COS-7 cells were transfected by electroporation with either empty pcDL-SRα296 vector (18) or pcDL-SRα296 containing CD40L cDNA (ATCC 79814). Transfected COS-7 cells were harvested 46 h post-transfection and fixed with 0.5% paraformaldehyde (16). Membrane CD40L expression was assessed by fluorescence-activated cell sorting using soluble CD40-Fc (1). The fixed cell preparation was able to stimulate B cell proliferation and IgE production in a dose-dependent manner (data not shown).

B Cell Proliferation Assay—Purified tonsillar B cells were stimulated for 3 days with IL-4 and various concentrations of recombinant soluble CD40L, anti-CD40 antibody (0.1 μg/ml, B-B20, Serotec, Oxford, United Kingdom) or various number of fixed CD40L-transfected COS-7 cells. Proliferation was assessed by incorporation of [^{3}H]thymidine for 6 h (1–2).

B Cell Differentiation Assay—The assay was performed with purified tonsillar B cells stimulated with IL-4 plus anti-CD40 and recombinant soluble CD40L. Immunoglobulins E, G, A, and M were quantified by specific enzyme-linked immunosorbent assay as described previously (19).

Apoptosis Assay—Recombinant soluble CD40L and anti-CD40 were tested for the ability to rescue purified germinal center B cells from apoptosis as described (20).

The sucrose gradient was performed as described for TNF (21).

RESULTS AND DISCUSSION

Extracellular Domain of the Recombinant CD40L—The extracellular domain of human CD40L (sCD40L-EC, Tyr-45 to Leu-261) expressed in *E. coli*, was found as an insoluble protein. In addition to the full-length form (29 kDa), two smaller fragments of 18 and 14 kDa were found and identified by N-terminal sequencing as beginning at ENSFEMQ and SNNLVT, respectively (Figs. 1 and 2). The 18-kDa fragment of sCD40L-EC is probably derived by processing of the 29-kDa form by an endogenous *E. coli* protease, since the fragment is preceded by two lysines. The observed proportions of the 18-kDa fragment varied in an *E. coli* strain-dependent manner

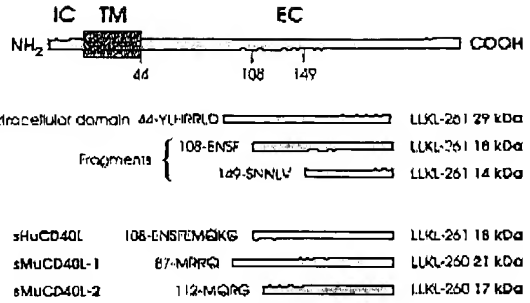
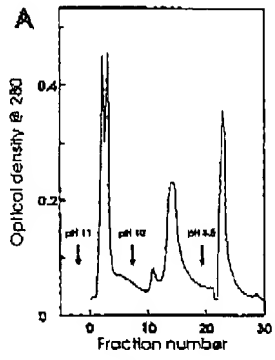


FIG. 1. **Schematic representation of extracellular domain of human and murine CD40L constructs and fragments.** The initial construct of the extracellular domain of the human CD40L encodes Tyr-44 to Leu-261 (shuCD40L-EC). Expression in *E. coli* gave the full-length (29 kDa) and two fragments of 18 and 14 kDa, which began at Glu-108 and Ser-149, respectively. Constructs were made encoding the human 18-kDa fragment of CD40L (sHuCD40L) and two murine constructs encoding Met-87 to Leu-260 (sMuCD40L-1) and Met-112 to Leu-260 (sMuCD40L-2), corresponding to the TNFα homologous domain, from Val-77 to Leu-233 (15).



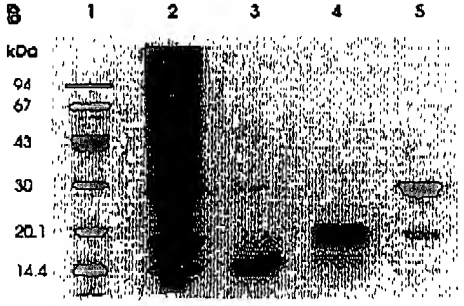


FIG. 2. **Purification of sCD40L-EC and its fragments.** Soluble CD40L-EC was purified on a Q-Hyper D anion exchanger as described under "Materials and Methods." *Panel A* shows elution profile. Fractions were analyzed by SDS-PAGE and purest fractions of 29, 18, and 14 kDa were each pooled and concentrated to 1 mg/ml. *Panel B* shows the SDS-PAGE analysis of the total washed pellet on *lane 2*, and the 14-, 18-, and 29-kDa shuCD40L protein pools on *lanes 3*, *4*, and *5*, respectively, obtained from elution at pH 11, 10, and 8.5 as shown in *panel A*. *Lane 1* corresponds to protein standards from Pharmacia.

(data not shown). The 14-kDa fragment may be due to an internal start as suggested by the presence of a Shine-Dalgarno-like sequence close to Met-148, 5′-AAA GGA TAC TAC ACC ATG. A partially purified mixture of these three proteins (Fig. 2*B*, *lane 2*), when added together with IL-10 or IL-4, was active in both a human B cell proliferation assay (data not shown) and an immunoglobulin switching assay using B cells derived from normal individuals and from patients with X-

TABLE I
Immunoglobulin production by HIGM patients' B cells with IL-4 and recombinant CD40L

Peripheral blood mononuclear cells were stimulated for 7 days with IL-4 alone or IL-4 plus recombinant CD40L protein mixture. Immunoglobulin concentrations were detected in supernatants by ELISA as described under "Materials and Methods."

Subjects	Unstimulated cells				Stimulated cells			
	IgM	IgG	IgA	IgE	IgM	IgG	IgA	IgE
	μg/ml				ng/ml			
Normal	65	90	20	<0.2	200	310	80	40
Patient	365	8	<1	<0.2	300	95	40	50

linked hyper-IgM syndrome (Table I and Ref. 9), indicating that the recombinant sCD40L-EC was able to trigger B cell activation through binding to the membrane CD40 on B cells. To understand which of the fragments of the extracellular domain of the CD40L trigger the biological activity, the recombinant extracellular CD40L form and the two fragments were further purified to homogeneity.

Purification, Biological, and Physical Characterization of the Active Recombinant Soluble Human CD40L—Protein purification was performed on a Q-Hyper D anion exchanger as described under "Materials and Methods." The sCD40L 14-, 18-, and 29-kDa fragments were eluted from the resin at pH 11, 10, and 8.5, respectively, at constant ionic strength (Fig. 2). The three purified sCD40L fragments were tested in the CD40L-CD40 binding and B cell proliferation assay over a concentration range of 1–100 μg/ml and 1–10 μg/ml, respectively (Fig. 3). The 18-kDa fragment was active in both assays, whereas the 29- and 14-kDa fragments failed to show any activity, indicating that the 18-kDa fragment is the active component of the mixture.

To further characterize the active extracellular domain of CD40 ligand, constructs of the 18-kDa soluble human CD40L (shuCD40L: Glu-108 to Leu-261) and the murine sCD40L, encoding homologous residues (smuCD40L-1: Met-87 to Leu-260) were made (Fig. 1). The purified 18-kDa shuCD40L and smuCD40L-1 confirmed that the 18-kDa CD40L fragment derived from the sCD40L-EC was the biologically active protein (Fig. 4). Purified 18-kDa shuCD40L and smuCD40L-1 were able to bind soluble CD40-Fc and stimulate B cell proliferation in combination with IL-4 (Fig. 4, *A* and *B*) with an IC_{50} of about 10 and 5 μg/ml, respectively. No signal was observed when CD40-Fc was not present in the binding assay (Fig. 4*A*) or when excess of sCD40L was added to the binding assay (data not shown). The 29-kDa extracellular domain (sCD40L-EC) and the 14-kDa fragment did not compete with 18-kDa shuCD40L for binding to CD40-Fc (data not shown). These results indicate that the 18-kDa domain of CD40L recognizes both the soluble and membrane-bound forms of CD40. A second protein construct of the murine CD40L (smuCD40L-2) that differs by five amino acids at the N-terminal end of the 18-kDa shuCD40L (Fig. 1, *108-ENSFE*), was found to be inactive at all concentrations tested (data not shown).

CD40 plays an important role in B cell differentiation and in the survival of germinal center B cells, as shown by the effects of cross-linking CD40 with agonistic anti-CD40 antibodies (22–23). Recombinant shuCD40L was tested for its ability to induce B cells to differentiate and to rescue germinal B cells from apoptosis. Addition of 1 and 5 μg/ml shuCD40L to purified human B cells allowed production of 28 ± 1 and 45.5 ± 2 ng/ml IgE (n = 3), respectively (Fig. 4*C*). Similarly, germinal center B cells were rescued from apoptosis in a dose-response manner by recombinant 18-kDa shuCD40L (Fig. 4*D*). These values were comparable to those obtained by addition of agonistic anti-CD40 antibodies (Fig. 4, *C* and *D*; Refs. 9, 22, and 28). Furthermore, a direct comparison of the 18-kDa shuCD40L with the membrane-bound full-length CD40L expressed in COS-7 showed that the two molecules displayed similar biological activity as shown by the B cell proliferation assay (Table II).

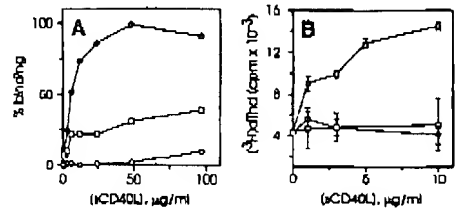


FIG. 3. **Biological activity of sCD40L-EC and its fragments.** *Panel A* shows the binding of shuCD40L-EC 29-kDa (○), 18-kDa (○), and 14-kDa (□) fragments to soluble CD40-Fc as described under "Materials and Methods." *Panel B* shows activation of human tonsillar B cells stimulated with IL-4 (100 units/ml) with or without increasing concentrations of the 29-kDa (○), 18-kDa (○), or 14-kDa (□) CD40L fragments. Both bioassays showed a saturable activity for 18-kDa fragment of shuCD40L-EC with EC_{50} = 5–10 μg/ml.

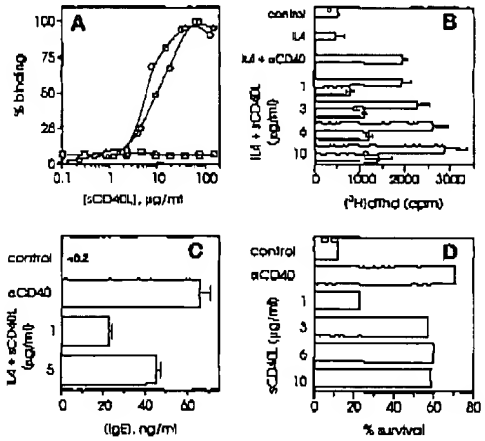


FIG. 4. **Biological activity of recombinant 18-kDa shuCD40L and smuCD40L.** The 18-kDa (shuCD40L) and murine (smuCD40L-1) recombinant extracellular domain constructs (Fig. 1) were expressed and purified as described under "Materials and Methods." *Panel A* shows the binding of CD40-Fc to 18-kDa shuCD40L (○) and smuCD40L-1 (○). Control without CD40-Fc (□) showed no detectable signal. *Panel B* shows B cell proliferation induced by stimulation with IL4 (100 units/ml) plus 18-kDa shuCD40L (*black bars*) or smuCD40L-1 (*open bars*) (n = 3). The 18-kDa shuCD40L was also able to induce B cells to differentiate and produce immunoglobulins (*panel C*) and rescue germinal B cells from apoptosis (*panel D*) (n = 3). The detection limit of IgE in *panel C* was around 0.2 ng/ml.

The soluble human CD40L corresponds to the region of TNFα homology (Val-77 to Leu-233). This region has been used to construct a three-dimensional model for CD40 ligand (15) which predicts a homotrimeric structure for CD40L. To investigate its quaternary structure, 18-kDa shuCD40L was analyzed by gel filtration; the molecule migrates with an apparent molecular mass of about 50 kDa. The 18-kDa shuCD40L isolated by gel filtration was active in the CD40L-CD40 binding assay (data not shown), indicating that the 18-kDa shuCD40L probably associates as a high order molecular complex. Further studies by sucrose gradient sedimentation studies suggest that the 18-kDa shuCD40L is homotrimeric (Fig. 5). Equal amounts of the 29-kDa (shuCD40L-EC) or of the 18-kDa (shuCD40L) fragment were overlaid in a 5–20% sucrose gradient and centrifuged for 40 h at 40,000 rpm in a SW 41 rotor. The 18-kDa sedimented as a unique molecular species, migrating with a molecular mass of 54.8 ± 0.6 kDa, which coincides with the

TABLE II

Stimulation of tonsillar B cells with anti-CD40 mAb, 18-kDa soluble CD40L, or COS-7 cells transfected with full-length CD40L

Purified tonsillar B cells (E^-) were incubated with IL-4 (100 units/ml) alone or in combination with anti-CD40 mAb (0.1 μg/ml, B-B20, Serotec), 18-kDa soluble CD40L (10 μg/ml), or transfected COS-7 cells fixed 48 h post-transfection with either empty pcDL-SRα 296 vector or same vector containing the full-length CD40L ($n = 3$). Proliferation assay and COS-7 cells transfection were done as described under "Materials and Methods."

B cell treatment	Thymidine incorporation
	cpm
Unstimulated	47 ± 19
IL-4	531 ± 181
IL-4 + anti-CD40	12,077 ± 765
IL-4 + 18-kDa soluble CD40L	21,820 ± 7310
IL-4 + control COS-7 transfectants	
5×10^4 cells	656 ± 254
1×10^4 cells	611 ± 929
IL-4 + CD40L COS-7 transfectants	
5×10^4 cells	42,517 ± 19,118
1×10^4 cells	28,148 ± 6476

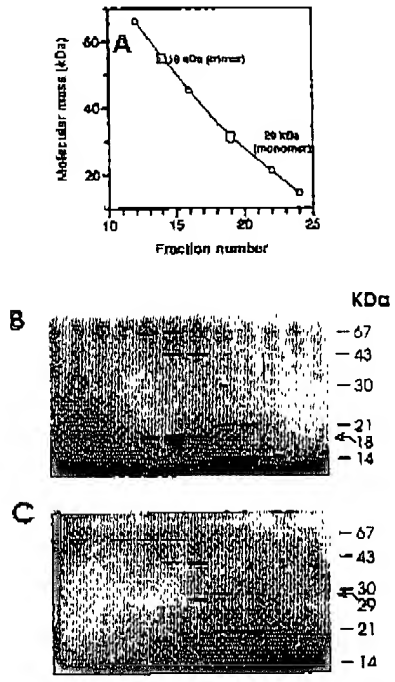


FIG. 5. **Sucrose gradient sedimentation of soluble CD40L.** One hundred μg of either 18-kDa shuCD40L or 29-kDa shuCD40L-EC were mixed with protein standards and layered onto a 5–20% sucrose gradient in PBS. After centrifugation for 40 h at 40,000 rpm in a SW 41 rotor, fractions of 300 μl were collected and analyzed by SDS-PAGE. *A*, molecular mass of globular protein standards was plotted against fraction number. Sedimentation points of the 18-kDa shuCD40L (54.8 ± 0.6 kDa) and the 29-kDa shuCD40L-EC (30.8 ± 2 kDa) are indicated. Calculated molecular mass of the two proteins was obtained from three independent gradients. *B* and *C*, fractions from representative gradients were separated by SDS-PAGE and silver-stained. Positions of the 18-kDa shuCD40L and 29-kDa shuCD40L-EC are indicated by *arrows*.

predicted trimeric conformation. In contrast, the 29-kDa sedimented with a molecular mass of 30.8 ± 2 kDa, suggestive of an extended monomeric form (Fig. 5). These results suggest that the trimeric conformation of the ligand may be required for binding to CD40. However, despite extensive refolding studies, we cannot rule out the possibility that the 29-kDa shuCD40L-EC fails to form trimers and to bind CD40 due to improper folding. A CD40L-CD8 chimera of the extracellular domain of human CD40L was reported to be active and trimeric (4). Thus, the CD8 domain may stabilize the trimeric form of CD40L-EC.

We have shown here that CD40L sequences corresponding to the TNF homology region can be expressed as a soluble trimeric molecule with biological activity. Its activity correlates with that of the membrane-bound CD40L; it can replace $CD40L^+$-T cells in the activation of B cells. These findings are supported by the fact that the soluble form of CD40L activates B cells derived from HIGM1 patients whose T cells lack active CD40L (Table I, Ref. 9). Our results suggest that, if a soluble form of CD40L exists *in vivo*, it could be active. Armitage *et al.* (24) described an activity from murine thymoma cell line (EL4) conditioned media that binds CD40 and stimulate human and murine B cells, supporting the possible existence of a soluble form of CD40L.

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